Glycoconjugate Journal 19, 319–323, 2003

The contribution of *in vivo* manipulation of gene expression to the understanding of the function of glypicans

Jorge Filmus

Sunnybrook and Women's College Health Sciences Centre, and Department of Medical Biophysics, University of Toronto, Canada

The name glypican identifies a family of heparan sulfate proteoglycans that are linked to the cell surface by a glycosylphosphatidylinositol anchor. Members of this family have been identified in *Drosophila*, zebrafish, and mammals. The interest in the study of glypicans has increased in the last few years as a result of the discovery that the glypican-3 gene (*GPC-3*) is mutated in an overgrowth and dysmorphic syndrome. Despite the increased interest, our knowledge about the function of glypicans is still limited, since the molecular basis for the role of glypican-3 in the regulation of body size remains unknown. The *in vivo* manipulation of glypican expression in lower organisms, however, has demonstrated that these proteoglycans can modulate cellular responses to Wnts and bone morphogenetic factors . Future studies should investigate whether the phenotype of *GPC-3*-deficient individuals is also due to altered modulation of cellular responses to these factors.
Published in 2003.

Keywords: **heparan sulfate proteoglycans, glypicans, mutant mice, overgrowth syndrome, wnt**

1. Introduction

Glypicans are heparan sulfate proteoglycans (HSPGs) that are attached to the exocytoplasmic surface of the plasma membrane by a glycosylphosphatidylinositol anchor [1,2]. Although the degree of amino acid homology between most glypicans is moderate [3], the location of 14 cysteine residues is conserved, suggesting that glypicans display a highly similar three-dimensional structure. Another shared feature of glypicans is the location of the heparan sulfate (HS) insertion sites, which seems to be restricted to the last 55 amino acids in the C-terminus, placing the HS chains close to the cell membrane [4].

To date six glypicans have been identified in mammals [3–10], two in *Drosophila* [11,12], and one in zebrafish [13]. During development, glypicans are generally expressed in a specific spatio-temporal fashion, suggesting that they play a role in morphogenesis [1,5,6,8,14–19]. The expression of some of these glypicans has been manipulated *in vivo* in various organisms and is shown in Table 1. In this mini-review we will summarize what we have learnt from these studies with regard to the function of this family of membrane-bound HSPGs.

Corresponding address: Sunnybrook and Women's College Health Sciences Centre, S211, 2075 Bayview Ave., Toronto, Ont. Canada, M4N 3M5. Tel.: (416)480-6100x3350; Fax: (416)480-5703; E-mail: jorge.filmus@swchsc.on.ca

2. Genetic manipulation of glypicans in *Drosophila*

The first glypican identified in *Drosophila* was *dally*. Although *dally*-null flies have not been isolated, hypomorphic mutants have an abnormal cuticle patterning in embryos, and display defects in several adult tissues, including the eye, antenna, wing, genitalia, and sensory organs in the wing and notum [11,20]. A detailed analysis of the phenotype of *dally* mutants and genetic interaction experiments have suggested tissue-specific roles of this glypican in the regulation of Wingless (Wg, a member of the Wnt family) and Decapentaplegic (Dpp, a member of the bone morphogenetic protein family) signaling pathways [21–23]. Specifically, the embryonic and adult wing phenothypes of *dally* mutants have been associated with reduced Wg signaling [21,22], while the abnormalities in the eye, genitals, and antenna are, at least in part, a consequence of reductions in Dpp activity [23]. The defects in the sensory organs of the wing have been associated with defects in both the Wg and the Dpp signaling pathways [20].

The second glypican identified in *Drosophila* is called *dally-like* (*dly*). Although the generation of *dly* mutants has not been reported yet, RNA interference experiments have shown that, as it was demonstrated for dally, inhibition of dly expression produces an epidermal patterning abnormality like that found in *wg* mutants [12], suggesting that this glypican is also required for optimal Wg signaling.

Table 1. *In vivo* manipulation of glypican expression

Species	*Glypican*	*Type of manipulation*	*Phenotype*	*Reference*
Drosophila	Dally	HM, EE RNAi	Altered wg and dpp signaling	[21,22]
	Dally-like	EE, RNAi	Altered wg signaling	[12]
Zebrafish	knypek	KO, EE	Altered convergent extension	[13]
Mice	GPC1	nr	nr	
	GPC2	nr	nr	
	GPC3	KO	Overgrowth, dysplastic kidneys, skeletal defects	[40–42]
	GPC4	nr	nr	
	GPC5	nr	nr	
	GPC6	nr	nr	

HM: Hypomorphic mutant; EE: ectopic expression; RNAi: RNA interference; KO: null mutant; nr: not reported.

The molecular basis for the regulatory activity of *Drosophila* glypicans on Wg and Dpp activity has not been uncovered yet. However, there is genetic evidence indicating that Wg signaling is also inhibited in *Drosophila* mutants with deficient heparan sulfate (HS) synthesis [12,24–26]. Since Wg and other members of the Wnt family can bind to heparin, it has been proposed that glypicans may directly bind to Wnts via their HS chains acting as co-receptors for these peptides [22]. This hypothesis is consistent with the finding that inhibition of HS sulfation blocks Wg activity in S2 *Drosophila* cells [27]. Although, cumulatively, it would appear then that glypicans function as positive regulators of Wnt signaling, it is important to note that there is also experimental evidence suggesting that the effect of glypicans on Wnt signaling is concentration-dependent. For example, ectopic expression of dly in the developing wing disc leads to an inhibition of Wg signaling [12]. Thus depending on the level of glypican expression during *Drosophila* development there may be a spatio-temporal variation in the regulation of Wg signaling.

Dpp plays a critical role in the establishment of dorsal/ventral polarity in *Drosophila*. Surprisingly, however, this polarity is not altered in *Drosophila* mutants with deficient HS synthesis, suggesting that at least in the embryo the HS of glypicans are not involved in the regulation of cellular responses to Dpp [24].

3. The role of the zebrafish glypican knypek

Convergent extension movements during gastrulation narrow the nascent embryonic axis, elongating it from head to tail. The establishment of the appropriate cell polarity is critical for convergent extension, and mutations of proteins that are necessary for the establishment of cell polarity also affect convergent extension. Large-scale genetic screens have established that components of the non-canonical Wnt signaling pathway play a critical role in convergent extension. Interestingly, it has been recently reported that knypek, a zebrafish protein that displays ~60% identity with glypican-4 and glypican-6, also regulates convergent extension movements [13]. *Knypek* mutants display impaired movements of convergent extension, which are associated with failure of mutant cells to acquire polarized cell morphology, and which generate a phenotype that is very similar to that of *Wnt-11* mutants. Moreover, double mutant and over-expression analyses show that knypek potentiates the non-canonical Wnt pathway [13]. Another morphogenetic process that is affected in *knypek* mutants is head cartilage elongation. Similar craniofacial abnormalities are observed in zebrafish *Wnt-5* mutants, which is another activator of the non-canonical Wnt pathway. Once again, as it was shown for dly in *Drosophila*, the effect of knypek on Wnt signaling is concentration-dependent, with high levels of knypek exerting a negative effect [13].

An important difference that has to be noted between the role of the *Drosophila* glypicans and knypek is that, whereas in the fly glypicans seem to act on the canonical Wnt pathway, only the non-canonical pathway is impaired in *knypek* mutants. Since each of these pathways seems to be regulated by particular Wnts [28], it can be proposed that there is some type of specificity in the interaction between glypicans and Wnts, and that the outcome of such interaction will depend on which pathway is regulated by the particular Wnt involved.

4. Genetic manipulation of glypican-3 in mammals

To date the only mammalian glypican whose expression has been manipulated *in vivo* is glypican-3. Undoubtedly the interest in the generation of *GPC-3*-null mice was triggered by the discovery that mutations of *GPC*-3 in humans generate the Simpson-Golabi-Behmel syndrome [29]. This syndrome is an X-linked disorder characterized by pre- and postnatal overgrowth, and a broad spectrum of clinical manifestations that vary from a very mild phenotype in carrier females, to infantile lethal forms in some males [30]. The clinical manifestations can include a distinct facial appearance, macroglossia, cleft palate, syndactyly, polydactyly, supernumerary nipples, cystic and dysplastic kidneys, congenital heart defects, rib and vertebral abnormalities, and umbilical/inguinal hernias [31–34]. In addition, there is an increased risk of developing pediatric tumors [31,35]. Most *GPC-3* mutations identified so far are point

mutations or small deletions [36–38]. Given the lack of correlation between patient phenotype and the location of the mutations, it has been proposed that the syndrome is caused by the lack of a functional glypican-3, with additional genetic factors being responsible for the intra- and inter-familial phenotypic variation [29]. The generation of glypican-3-deficient mice has provided strong support to this hypothesis [39–41]. These mice display several of the abnormalities found in the patients, including developmental overgrowth, early lethality, cystic and dysplastic kidneys, and skeletal defects. Detailed studies of the kidneys of the *GPC-3*-null mice demonstrated that from early stages of development there is a persistent increase in the proliferation rate of epithelial cells in the ureteric bud/collecting system [39]. This finding suggests that glypican-3 can act as a negative regulator of cell proliferation, which is obviously consistent with the general overgrowth in both the patients and the glypican-3-deficient mice.

Some of the abnormalities found in the patients, such as syndactyly and multiple nipples, suggest that glypican-3 can regulate cell survival in certain tissues during development. Experimental evidence supporting this hypothesis has been provided by the finding that glypican-3 can induce apoptosis in a cell-specific manner [42].

Some of the clinical manifestations of the patients are similar to those found in the Beckwith-Wiedemann syndrome [43]. Since over-expression of insulin-like growth factor II (IGF-II) is thought to be one of the contributing factors in this syndrome [44], it was originally proposed that glypican-3 negatively regulates IGF-II activity by competing for IGF-II binding with the IGF-I-receptor (the signaling receptor for IGF-II), and that *GPC*-3 loss-of-function mutations are equivalent to IGF-II over-expression [45]. Further support for this hypothesis was provided by the analysis of mice over-expressing IGF-II [46]. In addition to some of the phenotypic features of Beckwith-Wiedermann syndrome, IGF-II over-expressing mice display skeletal defects that are similar to those found in Simpson-Golabi-Behmel syndrome. However, the role of glypican-3 as a competitive inhibitor of IGF-II was put into question by the finding that it does not bind to IGF-II [47].

To investigate the relationship between glypican-3 and IGF-II in the regulation of body size, genetic interaction experiments were performed by breeding *GPC-3*-null and *IGF-II*-null mice [41]. If overgrowth in the absence of glypican-3 is only due to an increase in available IGF-II, the phenotype of *GPC-3/IGF-II* double knockouts should be identical to that of single *IGF-II* knockouts. However, this prediction was not fulfilled, since double mutant mice have a body size that is bigger than that of *IGF-II*-null mice. Similar observations were made with additional double mutants lacking *GPC-3* and *IGF-I* or IGF-I-receptor, indicating that glypican-3 does not act as a suppressor of body growth by altering only the interaction of the IGF-I-receptor with its ligands.

If glypican-3 regulates body size in an IGF/IGF-I-receptor-independent manner, how can we explain the similarities between Simpson-Golabi-Behemel syndrome and Beckwith-Wiedemann syndrome, and the Simpson-Golabi-Behemel syndrome-like features of IGF-II over-expressing mice? One possible explanation is that glypican-3 regulates a signaling pathway that converges with the IGF-signaling pathway downstream of ligand-receptor interaction. Since, as discussed above, glypican-3 has been shown to regulate the Wnt pathway both positively and negatively, could it be possible that this pathway is the one that converges with the IGF-II signaling system? Future studies will have to investigate this possibility.

It is also important to note that glypican-3 could also have an impact on other signaling pathways that do not converge with the IGF-signaling system. This would be consistent with the fact that Simpson-Golabi-Behemel patients and the *GPC-3*-null mice display severe kidney abnormalities that have not been observed in mice over-expressing IGF-II.

GPC-3-null mice have also been used to demonstrate that, like in *Drosophila*, glypican-3 can regulate cellular responses to bone morphogenetic proteins in specific tissues. This was achieved by breeding glypican-3-deficient mice with mice that were heterozygotes for bone morphogenetic protein-4 [40]. Interestingly, the offspring displayed polydactyly and rib malformations with high penetrance. These abnormalities were not observed in either parental strain. These results suggest that glypican-3 modulates cellular responses to bone morphogenetic protein-4 during limb patterning and skeletal developmental.

5. Future work

In vivo manipulation of glypican expression has already provided critical cues towards the understanding of the function of these proteoglycans. We expect that in the near future the generation of mice deficient in each of the members of the glypican family will provide valuable information regarding the specificity of glypican function. Moreover, the availability of such mice will allow the generation of double knockouts and, consequently, could unveil potentially overlapping functions of the various glypicans. It is important to note, however, that given the molecular complexity of glypicans, a complete understanding of glypican function will be required to complement the *in vivo* studies with biochemical and cell biology-based approaches.

Acknowledgment

I thank Howard Song for critically reviewing this manuscript.

References

1 Filmus J, Song HH, in *Glypicans*, edited by Iozzo RV. Proteoglycans, New York, Marcel Dekker, pp. 161–76.
2 Filmus J, Selleck SB, Glypicans: Proteoglycans with a surprise, *J Clin Invest* **108**, 497–501 (2001).

3 Veugelers M, De Cat B, Ceulemans H, Bruystens AM, Coomans C, Durr J, Vermeesch J, Marynen P, David G, Glypican-6, a new member of the glypican family of cell surface proteoglycans, *J Biol Chem* **274**, 26968–77 (1999).

4 Saunders S, Paine-Saunders S, Lander AD, Expression of the cell surface proteoglycan glypican-5 is developmentally regulated in kidney, limb, and brain, *Dev Biol* **190**, 78–93 (1997).

5 David G, Lories V, Decock B, Marynen P, Cassiman J, Van Den Berghe H, Molecular cloning of a phosphatidylinositol-anchored membrane heparan sulfate proteoglycan from human lung fibroblasts, *J Cell Biol* **111**, 3165–76 (1990).

6 Stipp CS, Litwac ED, Lander AD, Cerebroglycan: An integral membrane heparan sulfate proteoglycan that is unique to the developing nervous system and expressed specifically during neuronal differentiation, *J Cell Biol* **124**, 149–60 (1994).

7 Filmus J, Church J, Buick RN, Isolation of a cDNA corresponding to a developmentally regulated transcript in rat intestine, *Mol Cell Biol* **8**, 4243–9 (1988).

8 Watanabe K, Yamada H, Yamaguchi Y, K-glypican: A novel GPI-linked heparan sulfate proteoglycan that is highly expressed in developing brain and kidney, *J Cell Biol* **130**, 1207–18 (1995).

9 Veugelers M, Vermeesch J, Reekmans G, Steinfeld R, Marynen P, David G, Characterization of glypican-5 and chromosomal localization of human GPC5, a new member of the glypican gene family, *Genomics* **40**, 24–30 (1997).

10 Paine-Saunders S, Viviano BL, Saunders S, GPC6, a novel member of the glypican gene family, encodes a product structurally related to GPC4 and is colocalized with GPC5 on human chromosome 13, *Genomics* **57**, 455–8 (1999).

11 Nakato H, Futch TA, Selleck SB, The division abnormally delayed (dally) gene: A putative integral membrane proteoglycan required for cell division patterning during postembryonic development of the nervous system in Drosophila, *Development* **121**, 3687–702 (1995).

12 Baeg GH, Lin X, Khare N, Baumgartner S, Perrimon N, Heparan sulfate proteoglycans are critical for the organization of the extracellular distribution of Wingless, *Development* **128**, 87–94 (2001).

13 Topczewsky J, Sepich DS, Myers DC, Walker C, Amores A, Lele Z, Hammerschmidt M, Postlethwait J, Solnica-Krezel L, The zebrafish glypican knypek controls cell polarity during gastrulation movements of convergent extension, *Dev Cell* **1**, 251–64 (2001).

14 Litwack ED, Stipp CS, Kumbasar A, Lander AD, Neuronal expression of glypican, a cell surface glycosylphosphatidylinositol-anchored heparan sulfate proteoglycan, in the adult rat nervous system, *J Neurosci* **14**, 3713–24 (1994).

15 Ivins JK, Litwack ED, Kumbasar A, Stipp CS, Lander AD, Cerebroglycan, a developmentally regulated cell-surface heparan sulfate proteoglycan, is expressed on developing axons and growth cones, *Dev Biol* **184**, 320–32 (1997).

16 Pellegrini M, Pilia G, Pantano S, Lucchini F, Uda M, Fumi M, Cao A, Schlessinger D, Forabosco A, Gpc3 expression correlates with the phenotype of the Simpson-Golabi-Behmel syndrome, *Dev Dyn* **213**, 431–9 (1998).

17 Li M, Choo B, Wong Z-M, Filmus J, Buick RN, Expression of OCI-5/Glypican 3 during intestinal morphogenesis: Regulation by cell shape in intestinal epithelial cells, *Exp Cell Res* **235**, 3–12 (1997).

18 Veugelers M, Vermeesch J, Watanabe K, Yamaguchi Y, Marynen P, David G, GPC4, the gene for human K-glypican, flanks GPC3 on Xq26: Deletion of the GPC3-GPC4 gene cluster in one family with Simpson-Golabi-Behmel syndrome, *Genomics* **53**, 1–11 (1998).

19 Siebertz B, Stocker G, Drzeniek Z, Handt S, Just U, Haubeck H-D, Expression of glypican-4 in haematopoietic-progenitor and bone-marrow-stromal cells, *Biochem J* **344**, 937–43 (1999).

20 Fujise M, Izumi S, Selleck SB, Nakato H, Regulation of *dally*, an integral membrane proteoglycan, and its function during adult sensory organ formation of *Drosophila*, *Dev Biol* **235**, 433–48 (2001).

21 Lin X, Perrimon N, Dally cooperates with Drosophila Frizzled 2 to transduce Wingless signaling, *Nature* **400**, 281–4 (1999).

22 Tsuda M, Kamimura K, Nakato H, Archer M, Staatz W, Fox B, Humphrey M, Olson S, Futch T, Kaluza V, Siegfried E, Stam L, Selleck SB, The cell-surface proteoglycan Dally regulates Wingless signaling in Drosophila, *Nature* **400**, 276–80 (1999).

23 Jackson SM, Nakato H, Sugiura M, Jannuzi A, Oakes R, Kaluza V, Golden C, Selleck SB, dally, a Drosophila glypican, controls celular responses to the TGF-beta-related morphogen Dpp, *Development* **124**, 4113–20 (1997).

24 Baeg GH, Perrimon N, Functional binding of secreted molecules to heparan sulfate proteoglycans in Drosophila, *Curr Opin Cell Biol* **12**, 575–80 (2000).

25 Binari RC, Staveley BE, Johnson WA, Godovarti R, Sasisekharan R, Manoukian AS, Genetic evidence that heparin-like glycosaminoglycans are involved in wingless signaling, *Development* **124**, 2623–32 (1997).

26 Haerry TE, Heslip TR, Marsh JL, O'Connor MB, Defects in glucuronate biosynthesis disrupt Wingless signaling in Drosophila, *Development* **124**, 3055–64 (1997).

27 Reichsman F, Smith L, Cumberledge S, Glycosaminoglycans can modulate extracellular localization of the wingless protein and promote signal transduction, *J Cell Biol* **135**, 819–27 (1996).

28 Niehrs C, Solving a sticky problem, *Nature* **413**, 787–8 (2001).

29 Pilia G, Hughes-Benzie RM, MacKenzie A, Baybayan P, Chen EY, Huber R, Neri G, Cao A, Forabosco A, Schlessinger D, Mutations in GPC3, a glypican gene, cause the Simpson-Golabi-Behmel overgrowth syndrome, *Nature Genet* **12**, 241–7 (1996).

30 Neri G, Gurrieri F, Zanni G, Lin A, Clinical and molecular aspects of the Simpson-Golabi-Behmel syndrome, *Am J Med Genet* **79**, 279–83 (1998).

31 Hughes-Benzie RM, Pilia G, Xuan JY, Hunter AGW, Chen E, Golabi M, Hurst JA, Kobori J, Marymee K, Pagon RA, Punnett HH, Schelley S, Tolmie JL, Wohlferd MM, Grossman T, Schlessinger D, Mackenzie AE, Simpson-Golabi-Behmel syndrome: Genotype/phenotype analysis of 18 affected males from 7 unrelated families, *Am J Med Genet* **66**, 227–34 (1996).

32 Garganta CL, Bodurtha JN, Report of another family with Simpson-Golabi-Behmel syndrome and a review of the literature, *Am J Med Genet* **44**, 129–35 (1992).

33 Behmel A, Plochl E, Rosenkranz W, A new X-linked dysplasia gigantism syndrome: Identical with the Simpson dysplasia syndrome? *Hum Genet* **67**, 409–13 (1984).

34 Golabi M, Rosen L, A new X-linked mental retardation overgrowth syndrome, *Am J Med Genet* **17**, 345–58 (1984).

35 Lapunzina P, Badia I, Galoppo C, De Matteo E, Silberman P, Tello A, Grichener J, Hughes-Benzie R, A patient with Simson-Golabi-Behmel syndrome and hepatocellular carcinoma, *J Med Genet* **35**, 153–6 (1998).

36 Xuan JY, Hughes-Benzie RM, Mackenzie AE, A small interstitial deletion in the GPC3 gene causes Simpson-Golabi-Behmel syndrome in a Dutch-Canadian family, *J Med Genet* **36**, 57–8 (1999).
37 Hughes-Benzie RM, Hunter AGW, Allanson JE, Mackenzie AE, Simson-Golabi-Behmel syndrome associated with renal dysplasia and embryonal tumor: Localization of the gene to Xqcen-q21, *Am J Med Genet* **43**, 428–35 (1992).
38 Li M, Shuman C, Fei YL, Cutiongco E, Bender HA, Stevens C, Wilkins-Haug L, Day-Salvatore D, Yong SL, Geraghty MT, Squire JA, Weksberg R, GPC3 mutation analysis in a spectrum of patients with overgrowth expands the phenotype of Simpson-Golabi-Behmel syndrome, *Am J Med Genet* **102**, 161–8 (2001).
39 Cano-Gauci DF, Song H, Yang H, McKerlie C, Choo B, Shi W, Pullano R, Piscione TD, Grisaru S, Soon S, Sedlackova L, Tanswell AK, Mak TW, Yeger H, Lockwood GA, Rosenblum N, Filmus J, Glypican-3-deficient mice exhibit the overgrowth and renal abnormalities typical of the Simpson-Golabi-Behmel syndrome, *J Cell Biol* **146**, 255–64 (1999).
40 Paine-Saunders S, Viviano BL, Zupicich J, Skarnes WC, Saunders S, Glypican-3 controls cellular responses to Bmp4 in limb patterning and skeletal development, *Dev Biol* **225**, 179–87 (2000).
41 Chiao E, Fisher P, Crisponi L, Deiana M, Dragatsis I, Schlessinger D, Pilia G, Efstratiadis A, Overgrowth of a mouse model of the Simpson-Golabi-Behmel syndrome is independent of IGF signaling, *Dev Biol* **243**, 185–206 (2002).
42 Duenas Gonzales A, Kaya M, Shi W, Song H, Testa JR, Penn LZ, Filmus J, OCI-5/GPC3, a glypican encoded by a gene that is mutated in the Simpson-Golabi-Behmel overgrowth syndrome, induces apoptosis in a cell line-specific manner, *J Cell Biol* **141**, 1407–14 (1998).
43 Weng EY, Mortier GR, Graham JM, Beckwith-Wiedemann syndrome, *Clin Pediat* **34**, 317–26 (1995).
44 Weksberg R, Squire JA, Molecular biology of Beckwith-Wiedemann syndrome, *Med Pediatric Oncol* **27**, 462–9 (1997).
45 Weksberg R, Squire JA, Templeton DM, Glypicans: A growing trend, *Nature Genet* **12**, 225–7 (1996).
46 Eggenschwiler J, Ludwig T, Fisher P, Leighton PA, Tilghman SM, Efstratiadis A, Mouse mutant embryos overexpressing IGF-II exhibit phenotypic features of the Beckwith-Wiedemann and Simpson-Golabi-Behmel syndromes, *Genes & Develop* **11**, 3128–42 (1997).
47 Song HH, Shi W, Filmus J, OCI-5/rat glypican-3 binds to fibroblast growth factor-2 but not to insulin-like growth factor-2, *J Biol Chem* **272**, 7574–7 (1997).